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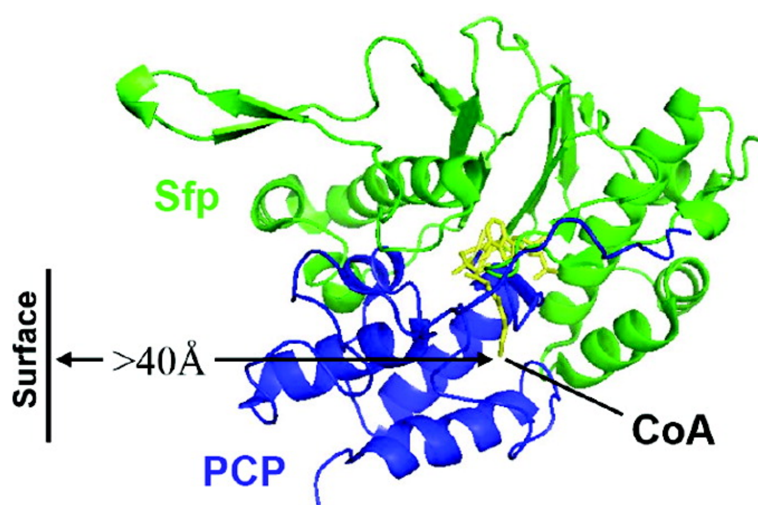

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Direct Site-Selective Covalent Protein Immobilization Catalyzed by a Phosphopantetheinyl Transferase

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Abstract: Immobilization of proteins onto solid supports is important in the preparation of functional protein microarrays and in the development of bead-based bioassays, biosensors, and industrial biocatalysts. In order to generate the stable, functional, and homogeneous materials required for these applications, attention has focused on methods that enable the efficient and site-specific covalent immobilization of recombinant proteins onto a wide range of platforms. To this end, the phosphopantetheinyl transferase Sfp was employed to catalyze the direct immobilization of recombinant proteins bearing the small, genetically encoded ybbR tag onto surfaces functionalized with CoA. Using mass spectrometry, it was shown that the Sfp catalyzes immobilization of a model acyl carrier protein (ACP) onto CoA-derivatized PEGA resin beads through specific covalent bond formation. Luciferase (Luc) and glutathione-*S*-transferase (GST) ybbR-fusion proteins were similarly immobilized onto PEGA resin retaining high levels of enzyme activity. This strategy was also successfully applied for the immobilization of the ACP, as well as ybbR-Luc, -GST, and -thioredoxin fusion proteins, on hydrogel microarray slides. Overall, the Sfp-catalyzed surface ligation is mild, quantitative, and rapid, occurring in a single step without prior chemical modification of the target protein. Immobilization of the target proteins directly from a cell lysate mixture was also demonstrated.

Introduction

The immobilization of proteins onto solid supports has become increasingly important, particularly in the generation of high-density protein microarrays for functional proteomics, including high-throughput analysis of protein−protein, protein−small molecule, and protein−nucleic acid interactions.[1] Despite this, the technical challenges associated with the generation of protein microarrays have prevented their widespread application. In addition, protein immobilization is important in other applications ranging from drug screening, diagnostics, and biosensing[1g,2] through to fundamental studies in single-molecule enzymology.[3] Protein immobilization methodology is also important in developing biocatalysts for industrial processes where there is a need to conserve or recycle commercially valuable enzymes.[4]

Early methods for protein immobilization were far from ideal, as they relied on nonspecific reactions such as the attachment of amino groups on the protein surface with aldehyde-functionalized solid supports.[1a] Such methods not only require a pure sample of each protein to avoid co-immobilization of protein impurities but also lead to a population of heterogeneously orientated proteins. Noncovalent protein immobilization using biotin−avidin,[5] anti-GST antibody,[1b] or His_6-Ni[1c,d] interactions has also been explored. However, the presence of avidin or antibodies on the surface could interfere with some assays, while His_6-Ni interactions are unstable to some experimental conditions. As a result, recent efforts have focused on site-specific covalent methods of protein immobilization.[6] These include the fusion of the protein of interest and a "capture" protein that reacts to form a covalent adduct with a substrate immobilized on a surface.[7] While this approach works well, protein fusion could affect the folding or function of some

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proteins of interest, and the co-immobilized capture proteins may also affect subsequent assays. Alternatively, protein *trans*-splicing, between a surface-immobilized *C*-intein and target protein-fused *N*-intein, can be used to effect "traceless" protein immobilization.[8] Finally, orthogonally reactive functional groups (e.g., azides or alkynes) can be introduced into proteins, using expressed protein ligation (EPL)[9] or protein farnesyltransferases,[10] allowing Staudinger or "click" ligation reactions with appropriately functionalized surfaces. While promising, these chemoenzymatic approaches[9,10] involve multiple steps and are restricted to immobilization via the *C*-terminus.

In this paper, a method is described that can be used to covalently immobilize proteins of interest site-specifically in a single, efficient enzymatic ligation step. This method utilizes the notable protein labeling technology developed by Walsh *et al.*[5a,11,12] and relies on Sfp, or a related phosphopantetheinyl transferase (PPTase) enzyme, to catalyze a reaction between coenzyme A (CoA) covalently immobilized on a solid surface and the protein of interest which contains a natural or engineered phosphopantetheinylation site (Figure 1). In nature, Sfp from *Bacillus subtilis* catalyzes the post-translational modification of an active-site Ser residue of *apo*-peptidyl carrier proteins (PCPs), resulting in *holo*-PCPs possessing phosphopantetheine prosthetic groups.[13] The utility of Sfp has been exploited to transfer a wide range of molecules, conjugated to the thiol moiety of CoA, onto various carrier proteins.[14] More recently, Sfp and related PPTases have also been used to label fusion proteins with fluorophores or biotin,[5a,11] the latter of which has enabled noncovalently immobilized protein arrays to be printed onto avidin-coated slides.[5a] The broader utility of the phosphopantetheinylation approach, compared with other methods, was demonstrated with the discovery of short peptide tags, including ybbR, which can be fused to internal loops as well as the *N*- or *C*-termini of a protein of interest while remaining a substrate for Sfp and related PPTases.[12]

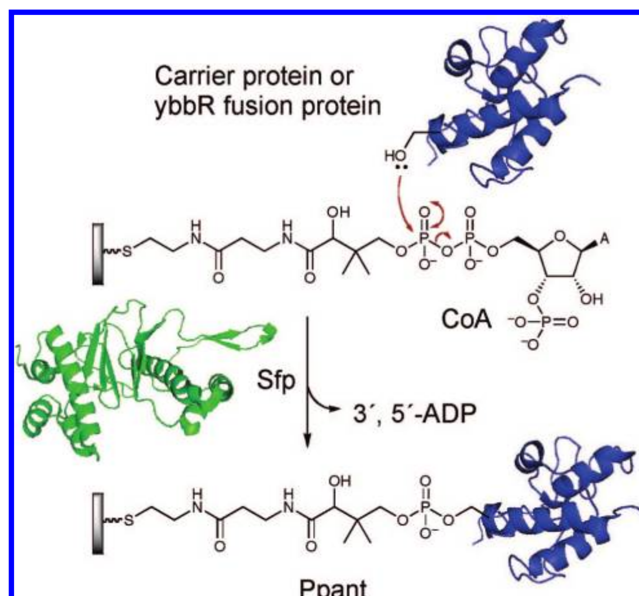

Figure 1. Strategy for site-specific protein immobilization using surface-immobilized CoA as a substrate to ligate a protein of interest fused to a carrier protein or possessing a helical peptide domain (e.g., ybbR), catalyzed by Sfp or a related PPTase enzyme.

Results and Discussion

Direct Immobilization of an Acyl Carrier Protein. Initially, the direct Sfp-catalyzed immobilization of an acyl carrier protein (ACP) onto PEGA1900 resin functionalized with CoA was studied (Figure 1). BtrI, an ACP involved in aminoglycoside biosynthesis, was selected as a model protein because it is known to be a good substrate for Sfp.[15] PEGA1900 was chosen as the support since it has flexible glycol amino-terminated chains that are up to 150 Å long, can accommodate biomolecules up to 70 kDa within the polymer matrix, and is amenable to multistep solid-phase synthesis.[16] Indeed, a molecular model of Sfp in complex with a PCP and CoA[17] indicates that the tether between CoA and the surface would need to be >40 Å to avoid steric interaction during ligation. Accordingly, the resin was first derivatized with the mild-acid-cleavable HMPB linker[18] and then acylated with FmocGly to give **1** (Scheme 1). Fmoc deprotection and coupling with the GMB linker produced a maleimide-derivatized resin **2**, which underwent a conjugate addition with the thiol group of CoA to give **3**. At this point, the HMPB linker was cleaved with 1% TFA, and the CoA adduct **4** was recovered from the resin. Analysis by HRMS (*m*/*z*: found $[M + H]^+$, 1008.1971, $C_{31}H_{48}N_9O_{21}P_3S$ requires 1008.1943) and ^1H NMR revealed a purity of >98%.

BtrI and Sfp were overproduced as described previously.[15] The CoA-derivatized resin **3** was then incubated with BtrI and Sfp in HEPES buffer supplemented with $MgCl_2$ at 37 °C for

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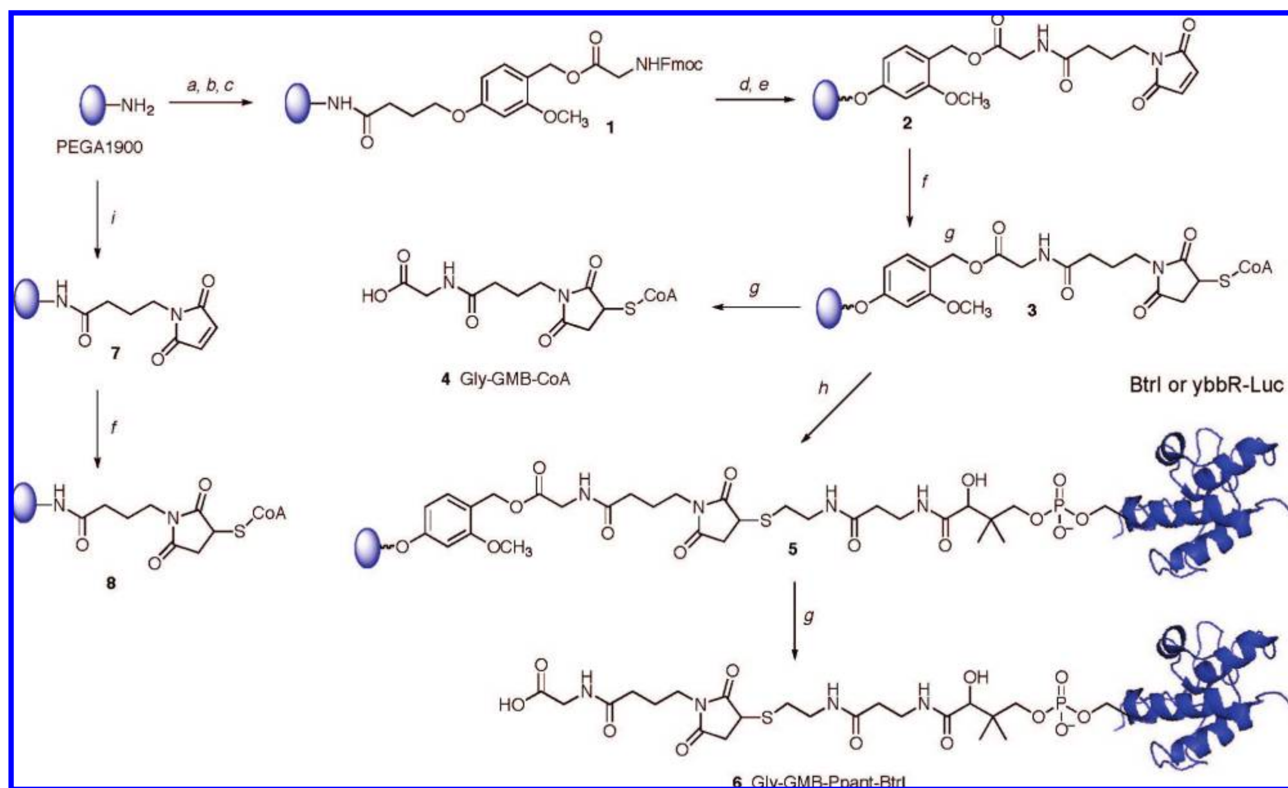
Scheme 1. Derivatization of PEGA1900 resin[a]



[a] Reagents and conditions: (a) HMPB, HOBt, DIC, DMF/DCM, 18 h × 2 cycles; (b) 5% N_2H_4, DMF, 1 h; (c) FmocGly, DIC, DMAP (cat.), DMF/DCM, 1.5 h × 2 cycles; (d) 20% piperidine, DMF, 5 min × 3 cycles; (e) GMB, DIC, HOBt, DMF/DCM, 16 h; (f) CoA, H_2O, 2.5 h; (g) 1% TFA, DCM, 5 min × 6 cycles then elute with H_2O; (h) BtrI, ybbR-GST or ybbR-Luc, Sfp, NaCl, $MgCl_2$, DTT, HEPES buffer (pH 7.0), 37 °C, 1 h; (i) GMB, DIC, HOBt, DMF/DCM, 6 h.

1 h. The resin was washed extensively with buffer and then treated with fluorescein-conjugated anti-polyHis antibodies. Fluorescence microscopy images of the resin **5**, after antibody treatment, clearly demonstrate the presence of the anti-polyHis-BtrI complex on the beads (Figure 2). In control experiments where the Sfp is omitted from the immobilization mixture, the fluorescence is only slightly higher than the background level that is evident when the resin **3** alone is treated with anti-polyHis antibodies. Although in several reports fluorescence microscopy of this type is presented as evidence of protein immobilization, it does not necessarily discriminate between specific covalent immobilization and nonspecific adsorption of proteins. Therefore, to demonstrate that the immobilization of BtrI was due to site-selective covalent bond formation, the HMPB linker of the resin **5** was cleaved, and the recovered protein was subjected to MALDI-MS (Figure 2). This shows that all the protein cleaved from the resin is 241 Da higher than *holo*-BtrI (BtrI-Ppant), which corresponds to the mass of the additional GMB-Gly linker ($C_{10}H_{12}N_2O_5$ requires *m/z* 240 Da). In addition, no other protein fragments or impurities are evident in the mass spectra. This confirms the identity of the protein as BtrI-Ppant-GMB-Gly **6** and provides further evidence that Sfp is able to catalyze the efficient and site-selective single-step ligation of an ACP with CoA immobilized on a solid support.

Immobilization and Activity of ybbR-Luciferase. The efficiency of this method for immobilization of functionally intact proteins was next tested using firefly luciferase (Luc). Accordingly, a synthetic gene encoding luciferase with an *N*-terminal His_6 tag followed by the 11mer ybbR peptide[12a] was cloned into the pET15b plasmid and overexpressed in *Escherichia coli*, producing the ybbR-Luc fusion protein. This fusion protein was purified by Ni-affinity chromatography and incubated with Sfp and CoA. MALDI-MS of the reaction mixture showed ybbR-Luc (found *m/z* 64 070, requires 63 962 Da) and the variant Ppant-ybbR-Luc (found *m/z* 64 381, requires 64 303 Da), which differ by 311 Da (Ppant calcd *m/z* 341), confirming successful phosphopantetheinylation. Following this, the CoA-derivatized PEGA resin **3** was incubated with ybbR-Luc and Sfp as before. UV absorption measurements of the reaction solution before and after immobilization indicate nearly quantitative (>95%) immobilization of ybbR-Luc. The resulting resin was washed and treated with the anti-polyHis antibodies and then subjected to fluorescence microscopy. As anticipated, the fluorescence was significantly higher than that observed for the resin from control experiments carried out in the absence of Sfp (see Supporting Information). Notably, the beads with the immobilized protein were larger than the controls, which was consistent with the immobilization of a hydrophilic protein resulting in increased swelling of the resin in aqueous solutions.

The functional integrity of the PEGA-immobilized Ppant-ybbR-Luc was next assessed by luminescence measurements in the presence of substrate luciferin and ATP (Table 1). First, the activity of the ybbR-Luc and the Ppant-ybbR-Luc protein in solution was determined, which revealed that phosphopantetheinylation of ybbR-Luc does not affect the activity of Luc. Subsequently, incubation of the resin-immobilized ybbR-Luc with luciferin and ATP also generated a high level of luminescence. On the basis of nearly quantitative levels of immobilization, the activity of the immobilized ybbR-Luc was found to be only slightly lower than that observed in solution. Equimolar amounts of resin derived from parallel control experiments following treatment with ybbR-Luc in the absence of Sfp show

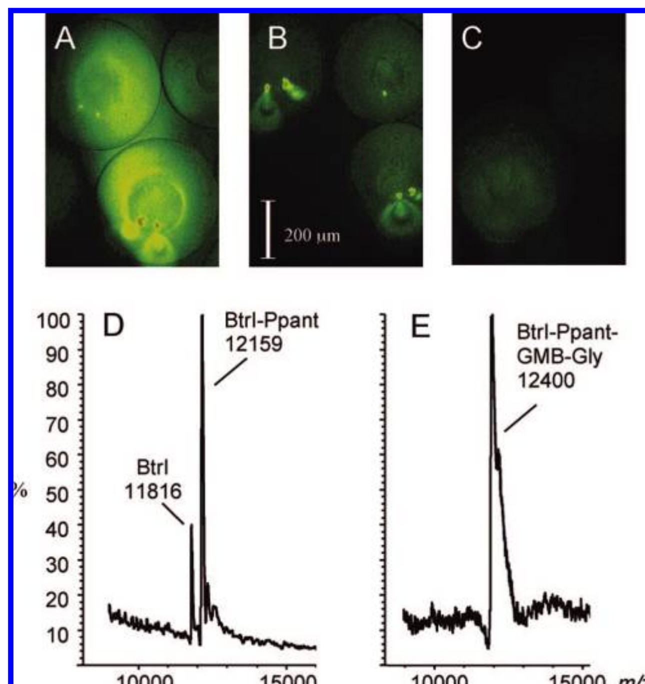


Figure 2. (Top) Fluorescence microscopy images of PEGA resin after treatment with (A) Sfp and BtrI, followed by fluorescein anti-polyHis antibody, which exhibits fluorescence of 125 au, measured from the center of the image; (B) BtrI alone (Sfp is omitted) and then anti-polyHis, which exhibits fluorescence of 48 au; (C) anti-polyHis antibody (BtrI and Sfp omitted), which exhibits fluorescence of 36 au. (Bottom) MALDI-MS of (D) BtrI and Sfp incubated with CoA, showing unreacted *apo*-BtrI (found *m*/*z* 11 816, requires 11 813 Da) and phosphopantetheinylated *holo*-BtrI (found *m*/*z* 12 159, requires 12 153 Da), and (E) BtrI-Ppant-GMB-Gly **6**, derived from cleavage of the HMPB linker of the BtrI immobilized on resin **5** (found *m*/*z* 12 400, requires 12 394 Da).

Table 1. Luminescence of ybbr-Luc in Solution and Immobilized

	luminescence/au (SD)	
	Luc in solution[a]	Luc immobilized[b]
ybbR-Luc[a]	13817 (3763)	
Ppant-ybbR-Luc[a]	16770 (2380)	
PEGA-CoA **3** + ybbR-Luc + Sfp[b]		12490 (1885)
PEGA-CoA **3** + ybbr-Luc (-Sfp)[c]		746 (253)
PEGA-CoA **3**[d]		36 (4)

[a] Solution-phase assays of Luc-ybbR or the phosphopantetheinylated Ppant-ybbR-Luc (3.90 nmol). [b] Resin assays: PEGA resin **3** (ca. 21.40 nmol surface-accessible CoA) was incubed with ybbR-Luc (3.90 nmol) and Sfp (0.5 nmol). [c] PEGA resin **3** treated as above except Sfp was omitted. [d] Untreated resin. Standard deviations (SD) are shown in parentheses.

only very low levels of luminescence under identical assay conditions. Thus, the method described here allows essentially quantitative immobilization of luciferase without any significant loss of activity, which further serves to demonstrate the efficiency and mildness of this methodology. This is particularly notable given that the nonspecific covalent immobilization of luciferase by traditional chemical means has been reported to be accompanied by significant loss of activity.[19] In addition, it is also possible to immobilize ybbR-Luc onto the CoA-derivatized resin directly from *E. coli* lysate using Sfp. In this case, luminometry shows significant albeit lower levels of luciferase activity on the resin.[20]

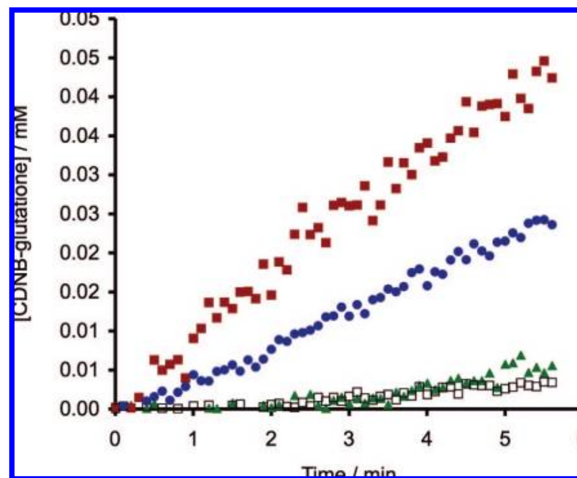


Figure 3. Initial reaction velocities (v_i) for the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione, catalyzed by equimolar ammounts of site-specifically (■) and nonspecifically (●) immobilized ybbR-GST. Control reactions with CoA-GMB-PEGA resin that was subjected to the same site-specific immobilization conditions, except Sfp was omitted (□), and with CoA-GMB-PEGA resin with no GST (▲) are also shown. The initial velocities for the reaction catalyzed by the site-specifically immobilized ybbR-GST fit a pseudo-first-order model, resulting in an apparent rate constant, *k*, of 0.093 min^{-1}. Interestingly, the nonspecifically immobilized ybbR-GST does not fit a simple first-order model. The atypical kinetics observed may be due to the heterogeneous orientation of the nonspecifically immobilized proteins and the fact that the many distinct protein species would exhibit different reaction kinetics.

Immobilization and Activity of ybbR-Glutathione-*S*-Transferase. In order to further study the enzymatic activity of proteins immobilized using this strategy, glutathione-*S*-transferase (GST) was immobilized onto PEGA resin. Here, the gene encoding GST was cloned into a variant of the pET30b vector possessing additional DNA sequence coding for the 11mer ybbR peptide. Overexpression of the resulting plasmid in *E. coli* produced the desired ybbR-GST fusion protein with *N*-terminal ybbR- and *C*-terminal His_6-tags. This protein was then isolated by Ni-affinity chromatography. Additionally, in order to simplify the preparation of CoA-bearing PEGA resin, the HMPB-Gly linker and related steps were omitted, giving CoA-GMB-PEGA (**8**, Scheme 1). The ybbR-GST was then conjugated to the resin **8** under essentially identical conditions as those used for the ybbR-Luc, and the rate of reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione catalyzed by ybbR-GST was measured with known amounts of protein-bearing PEGA resin (Figure 3). Using the standard literature UV assay for GST,[21] the initial reaction velocity (v_i) for the reaction catalyzed by the site-specifically immobilized ybbR-GST was calculated to be 98.3 μmol min^{-1}. In addition, an equivalent

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(20) Luminescence assays of the resin after immobilization of ybbR-Luc from lysate demonstrated a significantly higher activity (410 au) compared to the controls with Sfp ommitted (36 au) and untreated resin (26 au). However, the luciferase activity resulting from the lysate was lower than the activity from the immobilization of purified ybbR-Luc (Table 1). This is expected, given that the concentration of luciferase in the lysate was lower and endogenous CoA or acetyl-CoA present in the cell lysate can also compete as a substrate for the Sfp. In addition, ybbR-Luc is unstable at room temperature, and significant variations in specific activity of ybbR-Luc were observed between separately overproduced batches of the enzyme.

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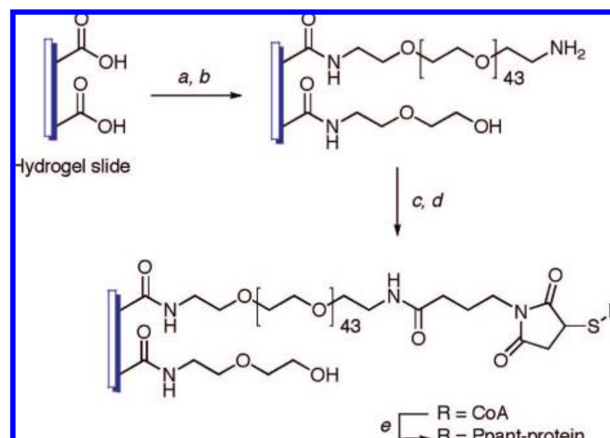
amount of ybbR-GST was nonspecifically immobilized via conjugate addition of the protein's surface thiols with the maleimide moieties of GMB-PEGA **7**.[22] Under identical assay conditions, the nonspecifically immobilized ybbR-GST gave a lower initial reaction velocity (v_i = 44.4 μmol min^{-1}). CoA-GMB-PEGA resin, which was subjected to the same immobilization conditions except with the Sfp omitted, and CoA-GMB-PEGA, which had not been exposed to any protein, both exhibited trace activities, with v_i = 7.6 and 4.9 μmol min^{-1}, respectively. These results therefore indicate that the GST that was immobilized site-specifically was higher in activity than the GST which was randomly attached to the PEGA resin. Furthermore, in the absence of the Sfp required to catalyze the immobilization reaction, almost no activity was detected above the baseline. These results are also in line with other reports which demonstrate that site-specific immobilization of proteins is superior to nonspecific immobilization.[9b,23]

Protein Immobilization onto Glass Microarray Slides. In order to demonstrate the generality of this approach and its application in the preparation of protein arrays, the immobilization of BtrI, Luc, GST, and also thioredoxin (Trx) onto glass microarray slides was next investigated. Accordingly, the gene encoding Trx was cloned into the pET30b plasmid, and the ybbR-Trx fusion protein, with *N*-terminal ybbR- and *C*-terminal His$_6$-tags, was overproduced and purified as described above. Initially microarray grade aminopropyl-functionalized slides were functionalized with CoA *via* a long polyoxyethylene spacer. While it was possible to immobilize proteins onto this surface, significant nonselective protein binding also was observed, and harsh denaturing washes were required to remove the noncovalently bound protein. Furthermore, blocking of the unreacted surface with bovine serum albumin (BSA) was required to prevent nonspecific adsorption of the labeling antibodies (see Supporting Information, Scheme S1 and Figure S5). As a result, hydrogel-coated slides (Nextrion Slide H) that were more resistant to nonspecific protein adsorption were employed. Accordingly, the hydrogel-carboxylate-coated slides, activated as succinimide esters, were derivativized with different ratios of polyoxyethylene bis-amine (with an average of 44 ethylene glycol units) and aminoethoxyethanol. Acylation with the GMB linker and conjugate addition of CoA gave slides with a range of different CoA surface concentrations (Scheme 2). Subsequent immobilization of BtrI onto the CoA-derivatized slides was then achieved with 3% mol equiv of Sfp. To detect the immobilized proteins, the slides were treated with horseradish peroxidase (HRP)-conjugated anti-polyHis antibodies and then fluorescently labeled with Cy5-tyramide.[24] From the fluorescence images of the slides following treatment with anti-polyHis antibodies, it was found that a mixture of just 1% long bis-amino glycol relative to the aminoethoxyethanol was suitable for the detection of immobilized BtrI (Figure 4A).

Immobilization of ybbR-Luc, ybbR-Trx, and ybbR-GST onto the 1% CoA-derivatized slides was similarly achieved with 3% mol equiv of Sfp. In all cases, the immobilization reactions were also found to proceed under ambient conditions, although somewhat slower: 5 h at room temperature, compared to 1 h at 37 °C. The presence of immobilized proteins on the surface

Scheme 2. Derivatization of Nextrion H Hydrogel Slides[a]



[a] Reagents and conditions: (a) DIC, HOSu, DMF, 2 h; (b) $H_2N(CH_2CH_2O)_{44}$-$CH_2CH_2NH_2$/$H_2N(CH_2)_2O(CH_2)_2OH$ (1:99), DMSO, 18 h; (c) GMB, DIC, HOSu, DMF, 2 h; (d) CoA, H_2O, 2.5 h; (e) BtrI, ybbR-Trx, ybbR-GST or ybbR-Luc, Sfp, NaCl, $MgCl_2$, DTT, HEPES buffer (pH 7.0), 37 °C, 1 h.

was demonstrated using the relevant anti-polyHis, anti-GST, or anti-Trx antibodies (Figure 4B). In the control positions, no proteins were detected on surfaces in the absence of CoA or in reactions where Sfp was omitted. In a separate experiment, it was also shown that ybbr-GST could be immobilized directly from the *E. coli* cell lysate (Figure 4C) using this method.

Conclusions

The phosphopantetheinyl transferase Sfp is able to catalyze the site-specific covalent immobilization of both ACP and ybbR fusion proteins onto solid supports derivatized with CoA. This single-step immobilization reaction is nearly quantitative, fast (1−5 h), mild (37 °C to room temperature, pH 7.0), and requires no prior chemical modification of the protein of interest or additional chemical steps. In addition, only small quantities of Sfp relative to the target protein (3% mol equiv) and of CoA relative to the bulk surface functionality (1% mol equiv) are required for effective immobilization of proteins onto hydrogel microarray slides. Notably, the Sfp-catalyzed immobilization of luciferase onto PEGA resin was achieved with no significant loss of activity, demonstrating that these conditions did not result in protein denaturation. In addition, it was shown that site-specifically immobilized GST possesses higher activity than GST which was nonspecifically immobilized onto PEGA resin. Given the orthogonal reactivity of Sfp and other PPTases for ybbR and related peptides,[12] it is envisaged that this method will allow for the fast, high-throughput immobilization of any functionally intact recombinant protein of interest directly from cell lysate, without interference from endogenous carrier proteins or host PPTase enzymes. The approach therefore offers some advantages over other existing methodologies for protein immobilization. This method could also be applied to other PEGA-based platforms, including the recently reported PEGA-coated microarray surfaces,[25] and would offer the opportunity to further streamline its application. Apart from its potential application on microarray platforms, the demonstration of immobilization on PEGA resin also points toward its use in

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(23) (a) Cha, T.; Guo, A.; Zhu, X.-Y. *Proteomics* **2005**, *5*, 416–419. (b) Kusnezow, W.; Hoheisel, J. D. *J. Mol. Recognit.* **2003**, *16*, 165–176.
(24) (a) Bobrow, M. N.; Litt, G. J. U.S. patent US5196306, 1993. (b) Khan, F.; He, M.; Taussig, M. J. *Anal. Chem.* **2006**, *78*, 3072–3079.
(25) Zourob, M.; Gough, J. E.; Ulijn, R. V. *Adv. Mater.* **2006**, *18*, 655–659.

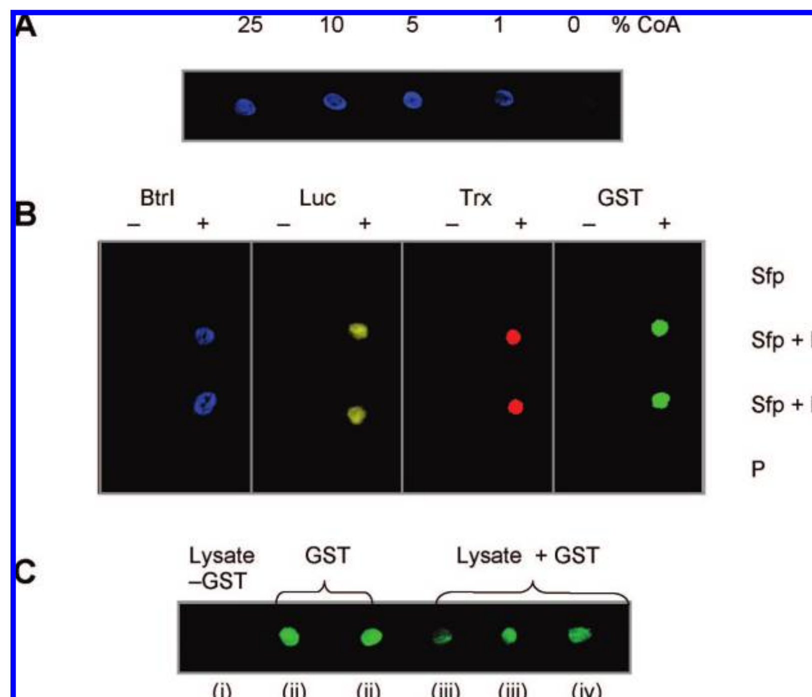

Figure 4. Fluorescence imaging of microarray slides. (A) Following immobilization of BtrI onto surfaces that differ in the concentration of CoA. (B) Following immobilization of target proteins BtrI, ybbR-Luc, ybbR-Trx, and ybbR-GST. Columns are labeled depending on the presence (+) or absence (−) of surface CoA. Along the top row, only Sfp is deposited. Rows labeled Sfp + P are spotted with Sfp and target proteins. Along the bottom row, only the target proteins are deposited. BtrI and Luc are imaged following treatment with anti-polyHis antibodies conjugated with horseradish peroxidase (HRP), resulting in Cy5-tyramide (blue) or Cy3-tryamide (yellow) deposition. Trx is imaged with the anti-Trx mouse antibodies followed by antimouse antibodies conjugated with TAMRA (red). GST is imaged with anti-GST antibodies conjugated with AlexaFluor 647 (green). (C) After immobilization of ybbr-GST from lysate: (i) Sfp and *E. coli* lysate with no GST; (ii) purified GST + Sfp; (iii) GST containing *E. coli* lysate with protease inhibitors, and (iv) GST containing *E. coli* lysate without protease inhibitors.

bead-based assay systems.[26] As far as we are aware, this is also one of the first examples of enzyme-catalyzed site-specific covalent protein immobilization that has been fully demonstrated to date.[27]

Experimental Section

Materials and Equipment. Amino-functionalized PEGA1900 resin with 300−500 μm diameter beads and a loading of 0.2 mmol/g was purchased from Polymer Laboratories (Church Stretton, Shropshire, UK) as an 8.3% suspension in MeOH. The Novabiochem HMPB linker and FmocGly were purchased from Merck Chemicals (Beeston, Nottinghamshire, UK); GMB from Molekula (Wimborne, Dorset, UK); polyoxyethylene bis-amine (MW_{av} = 2000) and CoA from Sigma-Aldrich (Gillingham, Dorset, UK). The Nexterion Slide H was purchased from Schott (Stafford, UK). All DMF used was of peptide synthesis grade from Rathburn Chemicals (Walkerburn, UK). All other chemicals were purchased from Acros.

The ybbR-Luc synthetic gene was purchased from Geneart AG (Regensburg, Germany). The pET28a-derived plasmids encoding BtrI and Sfp were gratefully received from Dr. Jonathan Spencer (University of Cambridge, UK).[15] The pGEX-6P-1 plasmids were supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK), and the Novagen pET48b plasmids were bought from Merck Chemicals. Chemicals and other materials used for the expression and purification of all proteins were purchased from Amersham Biosciences, Millipore, BioRad, Difco, Pharmacia, and Sigma. Rabbit polyclonal anti-polyHis antibodies labeled with FITC (200 μg/mL) and rabbit polyclonal anti-GST antibodies labeled with AlexaFluor 647 (100 μg/mL) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Novagen murine monoclonal anti-Trx IgG (1 mg/mL) from Merck Chemicals. The murine monoclonal anti-polyHis antibodies conjugated with horseradish peroxidise (12 mg/mL) and goat anti-mouse antibodies labeled with TAMRA (27 mg/mL) were purchased from Sigma-Aldrich. Tyramide signal amplification (TSA) Cy3 and Cy5 kits were purchased from PerkinElmer (Beaconsfield, Buckinghamshire, UK).

Fluorescence microscopy images were collected on a Carl Zeiss Axio Imager A1 with a FITC filter set and a Canon Powershot G6 digital camera with 10× magnification at the objective lens. All images were collected with a 20 s exposure time. MALDI-MS was performed with a Shimadzu Kratos Axima-CFR MALDI-TOF-MS with samples deposited by the “two-layer” method[28] in a sinapinic acid matrix. Luminescence was measured with an EG&G Berthold LB9507 Luminat luminometer. UV−vis kinetic measurements for GST[21] (Figure 3) were performed on a Varian Cary 400 spectrophotometer and the data processed using OriginPro version 8 data analysis and visualization software. Microarray images were acquired with an Amersham Biosciences Typhoon 8600 Variable Mode Imager in fluorescence mode using the appropriate excitation and filter settings for each fluorophore.

PEGA1900-HMPB. Amino PEGA1900 resin methanolic suspension (75 μmol amine sites) was washed with DCM (5 mL × 6) and drained. HMPB (54 mg, 225 μmol) and HOBt hydrate (38 mg, 248 μmol) were dissolved in DMF (1 mL) and DCM (1 mL). DIC (35 μL, 224 μmol) was added, and the mixture was allowed to activate for 5 min and then added to the DCM-washed resin. The mixture was shaken for 16 h, drained, and washed with DMF (5 mL × 5) and DCM (5 mL × 5). The resin was subjected to the

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(27) Transglutaminase has been shown to catalyze protein immobilization: Tanaka, Y.; Tsuruda, Y.; Nishi, M.; Kamiya, N.; Goto, M. *Org. Biomol. Chem.* **2007**, *5*, 1764–1770. However, the selectivity and utility of this approach have yet to be fully established.

(28) Dai, Y.; Whittal, R. M.; Li, L. *Anal. Chem.* **1999**, *71*, 1087–1091.

resin ninhydrin test,[29] and if a positive result was returned, the coupling reaction was repeated once. A solution of 5% $N_2H_4 \cdot H_2O$ v/v in DMF (3 mL) was added to the resin and shaken for 1 h. The resin was washed with DMF and DCM as described above.

PEGA1900-HMPB-Gly-Fmoc (1). Fmoc-Gly (56 mg, 188 μmol) was dissolved in DMF (250 μL) and DCM (1 mL), DIC (14.5 μL, 93 μmol) was added, and the mixture was allowed to activate for 5 min. This was added to the DCM-swollen HMPB resin (75 μmol), followed by DMAP (1 mg in 1 mL of DCM), and the entire mixture was shaken for 1.5 h. The resin was drained and washed with DMF (5 mL × 5) and DCM (5 mL × 5). A small sample of resin (ca. 5 mg) was subjected to the quantitative Fmoc test[30] and gave a loading of 0.16 mmol/g. (The coupling should be repeated if the conversion is <70% of the initial loading of the resin.)

PEGA1900-HMPB-Gly-GMB (2). The Fmoc-capped resin **1** (74 μmol) was mixed with 20% v/v piperidine in DMF (4 mL), shaken for 5 min, and drained. This was repeated for a total of three cycles, and the resin was washed with DMF, DCM, DMF, and DCM (5 mL × 5 each solvent in sequence). GMB (28 mg, 153 μmol) and HOBt hydrate (26 mg, 170 μmol) were dissolved in DMF (1 mL) and DCM (1 mL), DIC (24 μL, 153 μmol) was added, and the mixture was allowed to activate for 5 min. This solution was added to the resin, shaken for 16 h, and then washed with DMF (5 mL × 5) and DCM (5 mL × 5). The resin gave a negative result to the ninhydrin test and could be stored swollen in DCM for at least 6 months at room temperature.

PEGA1900-GMB (7). An amino PEGA1900 resin methanolic suspension (38 μmol amine sites) was washed with DCM (20 mL × 5) and drained. GMB (14 mg, 75 μmol) and HOBt hydrate (13 mg, 85 μmol) were dissolved in DMF (1 mL) and DCM (1 mL). DIC (11.5 μL, 73 μmol) was then added, and the mixture was allowed to activate for 5 min. This solution was added to the resin, shaken for 6 h, and then washed with DMF (10 mL × 5), DCM (10 mL × 5), and MeOH (10 mL × 5). The resin gave a negative result to the ninhydrin test and was stored swollen in MeOH.

PEGA1900-HMPB-Gly-GMB-CoA (3) and PEGA1900-GMB-CoA (8). Maleimido resin **2** or **7** (2.14 μmol) was washed with MeOH (1 mL × 5) and deionized water (1 mL × 5) and then drained. Coenzyme A sodium salt (3.4 mg, 4.33 μmol) was dissolved in deionized water (50 μL), added to the wet resin, and allowed to incubate at room temperature for 2.5 h. The resin was drained and washed with deionized water (1 mL × 10).

CoA-GMB-Gly-OH (4). The CoA resin (1 μmol) was rinsed with water, MeOH, and DCM (1 mL × 5 for each solvent) and drained. An aliquot (100 μL) of 1% TFA v/v in DCM was added to the resin, the mixture was gently swirled for 5 min, and the eluant was collected. Another aliquot of TFA solution was added, and the cleavage reaction was repeated for a total of six cycles. The resin was rinsed with MeOH (200 μL × 5), the washings were collected and then rinsed with water (5 × 200 μL), and those washings were collected. The MeOH and water fractions were combined and evaporated under reduced pressure to give an off-white solid (1.0 mg) of the desired product, which was pure by NMR (see Supporting Information for numbering of **4**). NMR: (500 MHz, D_2O) δ_H 0.77 (3H, s, methyl), 0.90 (3H, s, methyl), 1.83 (2H, tt, J = 7.0 and 7.2 Hz,19″), 2.28 (2H, t, J = 7.2 Hz, 20″), 2.44 (2H, t, J = 6.5 Hz, 7″), 2.59−2.66 (1H, m, 17″), 2.74−2.81 (1H, m, 11″), 2.85−2.93 (1H, m, 11″), 3.23 (1H, dd, J = 9.0 and 19.0 Hz, 13″), 3.37 (2H, dt, J = 6.4 and 14.0 Hz, 10″), 3.44 (2H, t, J = 6.5 Hz, 6″), 3.49 (2H, t, J = 6.9 Hz, 18″), 3.55 (1H, dd, J = 4.5 and 9.7 Hz, 1″), 3.82 (1H, dd, J = 4.5 and 9.7 Hz, 1″), 3.92 (1H, s(br), 3″), 3.95−4.01 (3H, m, 13″ and 23″), 4.21 (2H, d, J = 2.7 Hz, 5′), 4.56 (1H, s(br), 4′), 4.65−4.85 (2H, m, 2′ and 3′), 6.19 (1H, d, J = 5.9 Hz, 1′), 8.40 (1H, s, 2), 8.66 (1H, s, 8). MS: *m/z* (ES+) 1008 (15%, $[M + H]^+$), 1030 (45%, $[M + Na]^+$), 1052 (85%, $[M - H + 2Na]^+$), 1074 (100%, $[M - 2H + 3Na]^+$), 1096 (80%, $[M - 3H + 4Na]^+$), 1118 (60%, $[M - 4H + 5Na]^+$), 1140 (15%, $[M - 5H + 6Na]^+$). HRMS: found 1008.1943, $[M + H]^+$ requires 1008.1971.

Preparation of Hydrogel Slides. Nexterion Slide H slides are moisture sensitive and are stored at −20 °C in sealed containers which must be allowed to equilibrate to room temperature before opening. Inappropriate handling may result in hydrolysis of the surface succinimide esters. However, hydrolyzed slides could be reactivated with the following procedure: HOSu (93 mg, 0.8 mmol) was dissolved in DMF (4 mL), and DIC (125 μL, 0.8 mmol) was added. Aliquots of this mixture were immediately coated onto the surfaces of the slides (1 mL/slide) and allowed to stand at room temperature for 2 h. The slides were washed with DMF (×5) and dried under a stream of N_2. These were then used immediately.

Preparation of Hydrogel Slides with Varying Degrees of Amino-PEG Functionalization at Specific Locations. Separately, 40 mM stock solutions of polyoxyethylene bis-amine and 2-(2-aminoethoxy)ethanol in DMSO were prepared, and the appropriate volumes of each were mixed to give mixtures of 1:99, 5:95, 10:90, and 25:75 of the bis-amine to hydroxy-amine (1 mL of each mixture). Each mixture was spotted onto the appropriate locations on a slide (0.3 μL/spot) and incubated in a sealed chamber at room temperature for 18 h. The slide was washed with DMF (×5) and dried under a stream of N_2. The entire surface of the slide was then coated with 2-(2-aminoethoxy)ethanol (1 mL, 40 mM in DMSO) for 18 h at room temperature to block the remaining sites. The slide was washed with DMF (×5) and dried under a stream of N_2.

Preparation of Hydrogel Slides with 1% Amine Functionalization over the Entire Surface. The entire surface of a slide was coated with a 1:99 mixture of bis- and hydroxy-amines in DMSO (1 mL, prepared as described above) and incubated in a sealed container for 18 h. The slide was then washed with DMF and dried as described above.

Attachment of GMB Linker to Amine-Functionalized Hydrogel Slides. This procedure was applied to both the slides with varying amino group percentages and those with 1% amino functionalization. HOSu (19 mg, 165 μmol) and GMB (20 mg, 160 μmol) were dissolved in DMF (200 μL), and DIC (25 μL, 160 μmol) was added. The mixture was allowed to activate for 10 min before additional DMF (1800 μL) was added. This diluted solution was then coated over the entire slide surface (500 μL each slide) and allowed to incubate for 2 h. The slide was then washed with DMF and dried as described above. This slide may be stored at room temperature for up to several weeks.

Chemoligation of CoA to GMB-Functionalized Hydrogel Slides. This procedure was applied to both the slides with varying GMB percentages and those with 1% GMB functionalization. CoA (either sodium or lithium salts may be used, 1 μmol) was dissolved in water (6 μL) and the solution spotted onto the desired locations on each slide (0.3 μL/spot). These were incubated for 2 h in a sealed chamber and then washed with water (×10) and dried under an N_2 stream. The slides were used immediately for protein immobilization.

PCR Amplification of Trx and GST Genes. PCRs using the primers shown below were carried out using the pGEX-6P-1 vector template for GST and the pET48b vector template for thioredoxin. The PCR products were gel purified and then cut with restriction endonucleases *Hin*dIII and *Xho*I. The restriction fragments were then ligated into a pET30b vector that contained the ybbR tag.

Gstfor	5′-aagcttatgtcccctatactaggttat-3′
Gstrev	5′-ctcgagttttggaggatggtcgcc-3′
Trxfor	5′-aagcttatgagcgataaaattattcacctg-3′
Trxrev	5′-ctcgagggccaggttagcgtcga-3′

Overproduction and Purification of Sfp, BtrI, ybbR-Luc, ybbR-Trx, and ybbR-GST from *E. coli*. For expression of proteins, the relevant plasmid was transformed into BL21(DE3)pLysS

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(30) Eichler, J.; Bienert, M.; Stierandova, A.; Lebl, M. *Pept. Res.* **1991**, *4*, 296–307.

cells, and overexpression was induced at $OD_{600} = 0.6$ with 1 mM IPTG for 4 h at 37 °C. The cells were collected by centrifugation, and the pellets were resuspended in 20 mM $KHPO_4$, pH 7.5, 0.5 M NaCl, 5 mM imidazole, and 10% (w/v) sucrose (20 mL). PMSF (1 mM) was added, and the cells were lysed with lysozyme (1 mg/mL, 1 h at room temperature). The cell extract was clarified by centrifugation at 37000*g* for 30 min. The supernatant was loaded onto a 5 mL HisTrap column (Amersham Pharmacia Biotech) loaded with Ni^{2+} ions and equilibrated in binding buffer (20 mM phosphate, 0.5 M NaCl, 5 mM imidazole, pH 7.5). The column was washed until the absorbance returned to baseline. Elution of proteins was performed by the use of a step gradient. The column was washed with a solution containing 60 mM imidazole for six column volumes and then with 200 mM imidazole for 10 column volumes. Fractions containing the protein were concentrated and buffer-exchanged into 50 mM HEPES, pH 7.5, with 50 mM NaCl (for BtrI, ybbR-Luc, ybbR-GST, and ybbR-Trx) or 200 mM NaCl (for Sfp) with Amicon centrifugal filter units (Millipore). Glycerol (10% v/v) was added before aliquoting and snap-freezing.

Immobilization of BtrI or ybbR-Luc on CoA-GMB-Gly-HMPB-PEGA1900. The CoA resin (165 nmol) was rinsed with 50 mM HEPES, 50 mM NaCl, pH 7 buffer (1 mL × 5). Separately, the protein of interest (6−15 nmol at 15−30 μM in 50 mM HEPES, 50 mM NaCl, pH 7.5; proteins from frozen samples which in addition contain up to 10% v/v glycerol can also be used), Sfp (1−3 nmol at 10−50 μM in 50 mM HEPES, 200 mM NaCl, pH 7.6 buffer; previously frozen Sfp which includes up to 10% v/v glycerol can also be used), and a solution of $MgCl_2$/DTT (0.1 M $MgCl_2$ and 0.5 M DTT, 3 μL per 500 μL of reaction mixture) were mixed. The reaction solution was added to the buffered resin and incubated at 37 °C for 1 h. The reaction solution was drained and the resin rinsed with 50 mM HEPES, 50 mM NaCl, pH 7 buffer (1 mL × 5).

Immobilization of ybbR-GST onto GMB-PEGA and CoA-GMB-PEGA Resin. For nonspecific immobilization, ybbR-GST (11.3 nmol at 11.3 μM in 50 mM HEPES, 200 mM NaCl, pH 7.5; proteins from frozen samples which in addition contain up to 10% v/v glycerol can also be used) was mixed with 50 mM HEPES, 200 mM NaCl, pH 7.0 (212 μL) and added to an aliquot of GMB-PEGA resin **7** (equivalent to 550 nmol of maleimide sites). The mixture was incubated at 37 °C for 1 h and the resin washed as described above. The site-specific immobilization of ybbR-GST onto CoA-GMB-PEGA (**8**), catalyzed by Sfp, was carried out as described above.

Immobilization of ybbR-Luc from Cell Lysate to CoA-GMB-Gly-HMPB-PEGA1900. The ybbR-Luc was expressed, the cells were lysed, and the lysate was clarified by centrifugation in the same manner as described above. The CoA resin (165 nmol) was rinsed with 50 mM HEPES, 50 mM NaCl, pH 7 buffer (1 mL × 5). The lysate (10 μL) was diluted with 50 mM HEPES, 50 mM NaCl, pH 7 buffer (990 μL) and mixed with Sfp (1 nmol at 10 μM in 50 mM HEPES, 200 mM NaCl, pH 7.6, with 10% v/v glycerol) and $MgCl_2$/DTT solution containing 0.1 M $MgCl_2$ and 0.5 M DTT (6 μL). This solution then was added to the washed resin and incubated for 1 h at 37 °C. The resin was then washed as described above.

Anti-polyHis Antibody Labeling of Proteins on Resin. The antibody stock solution (200 μg/mL) was diluted with phosphate-buffered saline pH 7.4 (PBS, Sigma Aldrich recipe) to a ratio of 1:200. A small number of resin beads were rinsed with PBS (0.5 mL × 5) and incubated with the diluted antibody solution (sufficient to cover the beads) at room temperature for 1 h. The resin was then drained, washed with 0.1% Tween 20 v/v in PBS (1 mL × 5), and examined by fluorescence microscopy.

Luminometry. Separately, solutions of the ATP/reaction buffer (100 mM Gly_2, pH 7.8, 1250 μL; 1 M aqueous $MgSO_4$, 150 μL; 100 mM ATP disodium salt hydrate, pH 7.5, 500 μL; deionized water, 3100 μL) and luciferin solution (luciferin 10 mM in MeOH, 80 μL; 100 mM Gly_2, pH 7.8, 1000 μL; deionized water, 1920 μL) were prepared.

The reaction buffer (350 μL) was added to the protein solutions (containing up to 3.90 nmol of protein in 50 mM HEPES, pH 7.5, with 50 mM NaCl) or resin beads (equivalent to 80 nmol of the original CoA resin) and loaded into the luminometer. The luciferin solution (100 μL) was added and the luminescence measured for 20 s after a 10 s delay to allow for mixing. All measurements were conducted at steady state, in duplicate and on two separate occasions (four measurements in total) and the values averaged.

Cleavage of BtrI-Pant-GMB-Gly-OH from Resin and MALDI Analysis. The attached protein was cleaved from the resin (12 nmol) using the same procedure as for the cleavage of CoA-GMB-Gly-OH. The residue was reconstituted with aqueous 0.1% TFA (2 μL) for MALDI-MS analysis.

CDNB Assay of ybbR-GST Immobilized on PEGA Resin. For each assay, 50 mM HEPES, 50 mM NaCl, pH 7 (490 μL), and 50 mM MES, pH 6 (490 μL), were added to aliquots of resin beads equivalent to 1.7 nmol of immobilized enzyme (calculated from the UV−vis absorbance of the reaction solutions pre- and post-immobilization reaction) in a quartz cuvette. The beads were allowed to settle to the bottom of the curvette, 500 mM reduced glutathione (10 μL) followed by 100 mM CDNB in EtOH (5 μL) were added, and the entire mixture was thoroughly mixed. The beads were allowed to settle to the bottom of the cuvette (10−20 s), and the UV−vis absorbance at 340 nm was measured at 10 s intervals for at least 5 min. The concentration of the CDNB-glutathione adduct formed was calculated and plotted against time. The data were then fitted against a pseudo-first-order kinetic model, and the first derivative was calculated. The v_i was determined by the value of derivative (i.e., the gradient) at $t = 0$.

General Procedure for Immobilization of Proteins onto 1% CoA-Functionalized Hydrogel Slides. This procedure was applied to both the slides with varying CoA percentages and the 1% CoA functionalization. The target protein (10 μL at 10−125 μM protein in 50 mM HEPES, 50 or 200 mM NaCl, pH 7; previously frozen protein solutions which include up to 10% v/v glycerol can also be used) was mixed with Sfp (10 μL at 10−15 μM in 50 mM HEPES, 200 mM NaCl, pH 7.6; previously frozen Sfp which includes up to 10% v/v glycerol can also be used), $MgCl_2$/DTT solution (0.2 μL, 0.1 M $MgCl_2$ and 0.5 M DTT in deionized water), and aqueous 25% v/v Tween 20 (0.4 μL). This mixture was then deposited at the appropriate locations on a CoA-functionalized slide and incubated either at room temperature for 5 h or at 37 °C for 1 h in a sealed humidified chamber. The slide was washed with PBS with 0.5% v/v Tween 20 (×5) and deionized water (×1) and then dried by a stream of N_2. In the control experiments, the omitted protein solutions were replaced with an equivalent volume of 50 mM HEPES, 50 mM NaCl, pH 7.

Immobilization of ybbR-GST in Cell Lysate onto 1% CoA-Functionalized Hydrogel Slides. The ybbR-GST was expressed in the same manner as described above, and the cell suspension was divided into three parts and treated with lysozyme as described above. To two of the parts was added either PMSF (1 mM) or Roche complete protease inhibitor cocktail (according to the manufacturer's instructions), while no inhibitors were added to the third. These mixtures were then left at room temperature for 45 min before being clarified by centrifugation at 37000*g* for 30 min.

Aliquots of each cell lysate portion (1 μL) were diluted with 50 mM HEPES, 50 mM NaCl, pH 7 buffer (1 μL with 19 μL of buffer, final concentration of ybbR-GST in diluted lysate solution estimated to be 12 μM). This solution was mixed with Sfp (10 μL at 13 μM in 50 mM HEPES, 200 mM NaCl, pH 7.6, with 10% v/v glycerol), $MgCl_2$/DTT solution (0.3 μL, 0.1 M $MgCl_2$ and 0.5 M DTT in deionized water), and aqueous 25% v/v Tween 20 (0.6 μL). This mixture was deposited on the desired locations on the CoA-functionalized slide, incubated at room temperature for 5 h, washed with PBS with 0.5% v/v Tween 20 (×5) and deionized water (×1),

and dried by a stream of N_2. In the control experiments, the lysate was replaced with a solution of purified ybbR-GST.

Fluorescent Imaging of Immobilized Proteins. For BtrI and ybbR-Luc, the supplied anti-polyHis antibody-HRP conjugate was diluted in PBS with 0.5% v/v Tween 20 to a ratio of 1:8000. An aliquot of this diluted antibody solution (800 μL) was coated over the entire surface of the slide and incubated at room temperature for 1 h. The slide was washed with PBS with 0.5% v/v Tween and water and dried as above. The relevant reconstituted Cy5- or Cy3-tyramide solution (10 μL) and TSA working solution (500 μL), both from the TSA kits, were mixed, coated over the slide, and incubated for 3 min. The slide was washed and dried as above.

For detection of ybbR-Trx, the supplied anti-Trx mouse antibody was diluted with PBS with 0.5% v/v Tween 20 to a ratio of 1:800. An aliquot of this (800 μL) was coated over the entire slide, incubated at room temperature for 1 h, and subsequently washed and dried as above. The supplied anti-mouse antibody-TAMRA conjugate was diluted with PBS with 0.5% v/v Tween 20 to a ratio of 1:64. An 800 μL aliquot was coated over the entire slide, incubated at room temperature for 1 h, and then washed and dried as above.

For detection of ybbR-GST, the supplied anti-GST antibody labeled with AlexaFluor was diluted with 0.5% v/v Tween 20 to a ratio of 1:100. An aliquot of this (800 μL) was coated over the entire slide, incubated at room temperature for 1 h, and subsequently washed and dried as above.

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Supporting Information Available: Additional data, figures, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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